

# Regulation of Antigenic Variation in *Giardia lamblia*

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parasite, immune evasion, RNA interference, surface antigen, gene expression, vaccine

## Abstract

Antigenic variation, a clonal phenotypic variation developed by microorganisms, involves the permanent switching of homologous, antigenically different cell surface molecules. In pathogenic microorganisms, antigenic variation is often described as a mechanism to evade the host immune system and therefore is responsible for the generation of chronic and/or recurrent infections. However, antigenic variation has also been involved in expanding host diversity and differential courses of the diseases. The intestinal protozoan parasite *Giardia lamblia* undergoes antigenic variation through the continuous exchange of approximately 200 variant-specific surface proteins. Here we review the principal issues regarding the significance of antigenic variation during *Giardia* infections, the particular features of the variant-specific surface proteins, and the current knowledge on the mechanisms that regulate this process, as well as the relevance of disrupting antigenic variation as a novel approach to design effective antiparasitic vaccines.

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## INTRODUCTION

One of the most remarkable characteristics of microorganisms is their extraordinary capability to adapt to changes in the environment (137). Unicellular organisms, in particular, are exceptionally capable of rapidly adapting their gene expression profiles to differentiate into evasive or resistant forms to survive extreme conditions (137).

Pathogenic bacterial (107), fungal (58), or protozoan (39, 83, 113, 123) microorganisms are exposed to hostile conditions during their passage between the environment, vectors, and hosts and develop extraordinary mechanisms to adapt to such conditions (47, 141). During an infection, however, survival of pathogenic organisms depends not only on their capacity to adapt to a new host but also on their capability to neutralize the innate and adaptive immune defenses generated by the infected individual (64, 89, 149).

Surface antigenic variation is a major evasion system developed by pathogenic microorganisms to maintain chronic infections under the continuous immune pressures generated by their hosts (34, 35, 64, 136). Studies of antigenic variation in several organisms found that they use different mechanisms to switch the expression of their variable surface antigens (34). However, regardless of the diverse mechanisms of antigenic variation, this phenomenon always requires (*a*) a family of homologous genes present in the genomes of these organisms encoding immunodominant, antigenically different surface molecules; (*b*) a mechanism(s) that guarantees the mutually exclusive expression of only one antigen at a time; and (*c*) a mechanism for reversibly switching the expression of these molecules in individual cells (34). Antigenic variation has been documented in a variety of parasitic microorganisms, including *Trypanosoma*, *Anaplasma*, *Plasmodium*, *Babesia*, *Neisseria*, *Borrelia*, *Pneumocystis*, *Mycoplasma*, *Candida*, and *Giardia* species (34). It is speculative, yet reasonable, that many other unicellular organisms might also undergo antigenic variation, as suggested by recent reports (14, 37) and by the sequencing of many microbial genomes (38), where the presence of homologous gene families encoding potential surface molecules has been identified (24, 26, 57, 78).

The flagellated, binucleated parasitic protozoan *Giardia lamblia* (syn. *G. duodenalis* or *G. intestinalis*), one of the most common and best known parasites of humans and domestic animals (2), also undergoes antigenic variation (2, 90–92). In addition, because of its particular biological characteristics (29), *Giardia* has intrigued microbiologists since Antony van Leeuwenhoek discovered this parasite in his own feces 300 years ago (2).

*Giardia* belongs to the earliest diverging branch of the Eukaryota (82, 126). For this reason, *Giardia* is considered an excellent model system to study evolutionary aspects of basic cellular processes (29). In addition to its biological relevance, *Giardia* is one of the leading causes of human intestinal disease worldwide; the most frequent cause of

### Antigenic variation:

a clonal phenotypic variation involving cell surface antigenic determinants

*Giardia lamblia*: an intestinal parasite of humans

defined waterborne outbreaks of diarrhea in developed countries; and a common cause of diarrhea in day care centers, institutionalized individuals, backpackers, and travelers (130). In Asia, Africa, and Latin America approximately 200 million people have symptomatic giardiasis, with some 500,000 new cases reported each year (2). In regions where basic sanitation is deficient, *Giardia* infections are almost universal in members of the population by the age of two (76). *Giardia* has been recognized as a reemerging infection and has been recently included in the “Neglected Diseases Initiative” by the World Health Organization (122).

Phylogenetic studies of numerous *Giardia* isolates have allowed the identification of seven *G. lamblia* assemblages (A to G); A and B are capable of infecting humans (81, 92, 102). Assemblages A and B infect many mammals, which then serve as reservoirs for human infections (129). The *Giardia* human assemblage A (represented by the WB isolate) does not infect mice, which can be infected naturally by *G. muris*. Assemblage B (represented by the GS/M isolate) infects humans and mice (81, 92, 102), but the recent report of the draft genome sequencing of the GS/M isolate suggests that this group can be classified as a different species when compared with the genome of the WB isolate (46).

*Giardia* has a simple life cycle consisting of an infective cyst and a vegetative trophozoite. Infection is transmitted by ingestion of cysts, which are passed in the feces (70). After excystation in the upper small intestine, flagellated trophozoites are released (49). Trophozoites are not invasive and proliferate attached to the surface of the intestinal epithelial cells by means of a sucking disk (2). Trophozoites are responsible for the clinical manifestations associated with the disease, which vary from asymptomatic infections to acute or chronic severe diarrhea (23). Some individuals are chronically infected without presenting symptoms of diarrhea but suffer malabsorption and long-term detrimental effects on growth and development (the last of which occurs primarily in children) (131). The host immune status influences the susceptibility to infection and the severity of

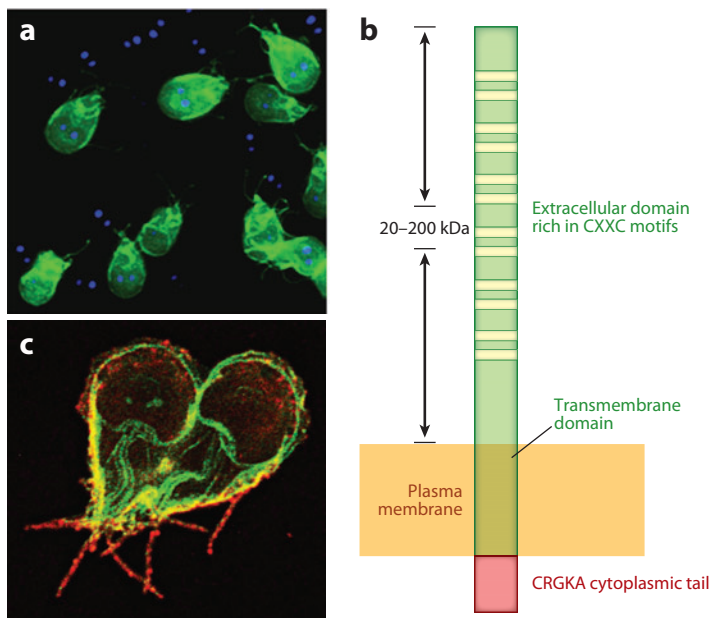
clinical signs. Children and the elderly are particularly susceptible to *Giardia* infections (23). Symptoms in humans typically occur one to two weeks after infection and last two to five days (91). Giardiasis seems to be self-limiting if the immune system of the host is fully developed, indicating that effective host defenses are able to control the disease. In some cases, however, chronic infections occur in the absence of any apparent immunodeficiency (91).

## ANTIGENIC VARIATION IN *GIARDIA LAMBLIA*

Antigenic variation was initially discovered in other pathogenic microorganisms in which symptoms of the disease correlated with peaks in parasitemia and differential surface antigen expression (35). By contrast, antigenic variation in *Giardia* was originally observed as a phenomenon occurring in vitro. The factors leading to the discovery of antigenic variation in *Giardia* were differences in surface-labeling patterns over time in trophozoites maintained in culture, variability in secretory/excretory products present in the culture medium of different populations of the parasite (3, 6, 90, 94, 98), and the development of specific monoclonal antibodies (mAbs) that recognize the surface of only some cells within a population (93) (**Figure 1a**). Therefore, antigenic variation in *Giardia* still occurs in the absence of any immune pressure.

Subsequent experiments demonstrated that *Giardia* antigenic variation also occurs during infections of humans and laboratory animals (7, 8, 96, 97). In the course of infection, proliferating trophozoites give rise to subpopulations that have switched to different surface antigens and therefore are able to elude host immune responses. These subpopulations can be then recognized by the immune system, but continuous switching to novel surface molecules prevents clearance of the infection (41, 87, 88, 90–92, 94).

Initial experiments in which antibodies were added to the culture resulted in aggregation and death of cells expressing a given surface protein specifically recognized by a mAb (93, 94).



**Figure 1**

Antigenic variation in *Giardia lamblia*. (a) Immunofluorescence assay on a population of *Giardia* trophozoites in culture labeled with a monoclonal antibody directed to a particular VSP (green) and counterstained with DAPI (blue) to label the nuclei of the parasites. Although the surface of some trophozoites is labeled with this antibody, many others are expressing a different VSP (only their nuclei are stained), indicating antigenic variation. Magnification 400 $\times$ . (b) Schematic of the structure of a *Giardia* VSP, showing the variable, cysteine-rich extracellular region, a single transmembrane domain, and a conserved cytoplasmic tail. (c) Confocal immunofluorescence assay using monoclonal antibodies directed to two different VSPs on trophozoites in which the RNAi pathway has been disrupted. Dual labeling (red and green, superimposition in yellow) can be observed in a pair of dividing trophozoites in which antigenic variation has been deregulated. Magnification 630 $\times$ . Abbreviations: RNAi, RNA interference; VSP, variant-specific surface protein.

By using mAbs as powerful tools, it was possible to clone trophozoites expressing a given antigen, demonstrating that *Giardia* species express only one antigen on their surface at a time, but that switching to another antigenically different surface antigen occurred once every 6 to 16 generations, depending on the isolate and the antigen (95). Nevertheless, simultaneous expression of two antigens on the surface of individual trophozoites was observed during switching, when dual labeling of the trophozoite surface with different mAbs lasted about 15 h (92), and during differentiation to and from cysts (28, 79).

## VARIANT-SPECIFIC SURFACE PROTEINS

Antigenic variation in *Giardia* involves variant-specific surface proteins (VSPs) (86). VSPs are key players in *Giardia* antigenic variation and are the focus of great controversy in the field because their characteristics may be related to regulation of the switching process (29). VSPs are cysteine-rich integral membrane proteins that possess a variable extracellular N-terminal region and a conserved C-terminal domain that includes a unique transmembrane region and a short, 5-amino-acid-long cytoplasmic tail (85, 101) (Figure 1b). VSPs form a thick coat on the parasite that represents the host-parasite interface (111). The density of VSPs in the plasma membrane generates a thick surface coat that might act as a physical barrier to prevent the immune system from accessing other components of the plasma membrane (91, 92, 111).

The first VSP was described using a partial mRNA sequence of VSP-A6, which is recognized by mAb 6A7 (3); two years later, the first complete sequence of another VSP, at that time called TSA417, was reported (48). Now, with the completion of the sequencing of the genomes from *Giardia* assemblage A clone WB (82), assemblage B clone GS/M-H7 (46), and assemblage E clone P15 (59), the sequences of previously identified and many novel VSPs are available. In silico studies led to the identification of approximately 200 *vsp* genes per genome with typical features of this protein family (variable, cysteine-rich N-terminal region, a conserved transmembrane domain, and the CRGKA cytoplasmic tail) (85). A previous report estimated that the VSP repertoire possesses about 133 to 151 VSP variants (101). However, the report of the first *Giardia* genome (82) indicates that *Giardia* encodes a repertoire of 235 to 275 *vsp* genes, which was confirmed by a recent analysis (4). In the latter study, 303 *vsp* genes were identified in the current assembly of the genome of the WB isolate. Of the 303 *vsp* genes, 228 were complete, whereas 75 were partial or incomplete sequences. These genes are clustered in groups of two to nine distributed

### Variant-specific surface protein (VSP):

an antigen involved in antigenic variation in *Giardia*

*vsp*: VSP-coding gene

all over the five chromosomes. Because of the similarity that exists between *vsp* upstream sequences, it has been proposed that these repertoires resulted from divergence and subsequent recombination events (146). This assumption is supported by the fact that of the 228 *vsp* genes estimated in the recent analysis, 10 were identical inverted gene pairs (4). In addition, some VSPs share some degree of homology and can be recognized even by the same mAb (28). Moreover, the study of antigenic variation using polyclonal antibodies yields confusing results because several VSPs may share common epitopes (113).

As with most other *G. lamblia* genes, *vsp* genes are believed to have no introns and their promoters are relatively short and have limited sequence conservation. The 3' untranslated region of *Giardia* genes, including *vsp* genes, also tends to be short, typically no longer than 30 nucleotides (nt) (1, 2, 92, 101). However, the presence of a candidate initiator element (Inr) consisting of PyAatgTT, where atg represents the initiator codon, was recently suggested (4). This initiator element is present in about 40% of the identified *vsp* genes of the WB genome, indicating the lack of homology of the 5' upstream regions of the *vsp* genes. In addition, *vsp* genes are not necessarily telomeric, and recombination associated with relocation to other regions of the genome has not been documented in contrast to what was found in other parasites that undergo antigenic variation (1, 2, 4).

The amino-terminal portion of the VSPs presents a high degree of variation among the different members of the family (ranging in size from 20 to 200 kDa), with variability in the number of CXXC motifs (where C is cysteine and X represents any amino acid) (2). The extracellular N terminus forms the interface between the parasite and the host and is the portion of the molecule recognized by specific antibodies generated during *Giardia* infections (85, 93). Moreover, the constitutive expression of amino-terminal hemagglutinin (HA)-tagged VSPs and the posterior identification of the localization of the chimeric protein using

anti-HA-specific antibodies showed that the complete surface of the trophozoites becomes labeled, confirming that the N-terminal region is extracellular and VSPs are type 1a integral membrane proteins (65). On the other hand, the C-terminal portion of the VSPs exhibits high conservation among the different family members, showing almost 90% identity in the amino acids of the transmembrane domain and a totally conserved 5-amino-acid cytoplasmic tail (CRGKA) (85).

Several posttranslational modifications have been reported in VSPs; however, due to the conflicting results that have been informed (reviewed in Reference 113), profound studies are necessary to determine the existence of these modifications and their possible relevance for the biology of *G. lamblia*.

The transport of newly synthesized VSPs to the plasma membrane is similar to that of higher eukaryotes. The presence of a recognizable signal peptide (10, 71) in the amino-terminal portion of the protein is enough to ensure the directional movement of the VSPs to the plasma membrane by the constitutive secretory pathway. Two reports present contradictory results regarding the transport of the VSPs when considering the conserved cytoplasmic tail. As reported, elimination or modification of the conserved amino acids of the VSP tail prevented the exit of newly synthesized VSPs from the endoplasmic reticulum (75). Conversely, other authors found that the elimination of the conserved cytoplasmic tail does not have any effect on normal VSP transport to the plasma membrane (133). There is no satisfactory explanation for these conflicting results.

On the basis of the results of immunogold transmission electron microscopy (77, 111), VSPs are present on the surface of the parasite, forming a continuous coat that covers the entire cell surface, including the flagella and the ventral disk, as well as the rough endoplasmic reticulum (site of synthesis) and the peripheral vacuoles (PVs), which seems to represent a primitive endosomal/lysosomal system that is located underneath the plasma membrane of

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PV: peripheral vacuole

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### RNA interference

(RNAi): a mechanism for degrading dsRNA in a sequence-specific manner

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the parasite (67). What is the reason for the presence of VSPs in the PVs? Are the PVs the site of VSP degradation?

An important issue in need of further investigation is how a VSP is replaced by another VSP during antigenic switching. This information is relevant not only for *Giardia*, but also for other organisms that undergo antigenic variation. Are the VSPs transported from the plasma membrane to the PVs for recycling, or are the VSPs released by specific cleavage, as suggested (108)? If they are endocytosed, how is the former VSP discriminated from the novel VSP if they have the same cytoplasmic tails? Are the suggested posttranslational modifications of the tail a signal for discrimination between both VSPs (132, 134)? If so, is there a signal that marks the former VSP before the newly synthesized VSP is incorporated into the plasma membrane? Or are the VSPs cleaved by specific proteases facing the cell exterior for release into the environment?

It is well known that VSPs appear in the culture medium (71, 98) and have been considered secretory/excretory products of *G. lamblia* that could be involved in diverting the immune system while the parasite switched its original VSP (91). Then, given that the cleavage sequence is the same for all VSPs, how can a protease differentiate among them (108)? Although immunofluorescence confocal microscopy and flow cytometry assays performed with mAbs specific to two different VSPs colocalized both proteins to the surface of individual cells (99, 134), whether different VSPs are located in different regions of the plasma membrane (132, 134) remains unclear. But again, whether the CRGKA tail is important for VSP switching, transport, or both, and how one VSP is replaced by another antigenically different VSP remain a mystery. Nevertheless, either the natural half-lives of the VSPs (involving degradation, release, or both) linked to differences in level of their synthesis or an active mechanism of exchange may be playing a role in this process. Further experiments are necessary to clarify this issue.

## MOLECULAR MECHANISMS INVOLVED IN ANTIGENIC VARIATION

For several years, many research groups have focused on elucidating the regulatory mechanisms that are responsible for antigenic switching in *Giardia* (92). However, these investigations were unable to clarify whether regulation takes place at the genomic, transcriptional, or posttranscriptional level (2).

In clones expressing only one VSP on their surface, it was found that the only transcript (mRNA) detected by Northern blotting was the one corresponding to the VSP that is expressed (5). This finding suggests transcriptional control, because of the 200 *vsp* genes only one seemed to be transcribed whereas the others remained silent. It was later demonstrated that all *vsp* genes are transcribed but that all but one are silenced by an RNA interference (RNAi)-like mechanism. This mRNA always corresponds to the variant expressed on the parasite surface (5, 85, 86). After VSP switching occurs, the original transcript disappears with the appearance of a distinct VSP mRNA (3, 85, 144, 147). It has been proposed that *Giardia* is a tetraploid organism and therefore possesses four copies of each allele of a *vsp* gene (17). It has been reported that only the *vsp* genes that possess tandem repeats can be recognized by alleles with a certain number of repeats, since it was observed that only one allele of the set is expressed at a given time (51, 144). For example, one allele from the VSP-A6 gene, when expressed at the level of protein, was lost in clones that do not express that variant (5). The presence of allele-specific expression of *vsp* genes suggests an epigenetic mechanism regulates surface antigen expression in *Giardia*, but no experimental evidence supports this idea (144–147).

During the process of trophozoite differentiation into cysts (encystation) or from cyst to trophozoites (excystation), VSP switching becomes evident in the isolate WB (79). This mechanism is not universal because after completion of the life cycle in the GS isolate the original VSP remains unchanged by

other immunologically different VSPs (128). However, it is also unclear whether antigenic switching occurs during differentiation or cell division, events intrinsically linked to both encystation and excystation processes (2).

Transcriptional control of constitutively expressed and regulated genes in *Giardia* is driven by small promoter sequences identified upstream of the translational start sites (148). Studies of these sequences suggest that the presence of AT-rich fragments can allow transcription initiation in *G. lamblia* (2). However, no VSP promoter sequence has been identified to date, and the upstream regions of the *vsp* genes do not show any putative sequence that can control transcription. Adam et al. (4) indicated that a fraction of *vsp* genes contain the initiator sequence PyAatgTT (see above). Nevertheless, no experimental data have been provided to determine whether only this fraction of the VSP repertoire containing initiator element motifs is active while the other fraction is not (4). All these results indicate that there is no consensus on the mechanisms that control/regulate VSP expression in *Giardia*.

Is VSP expression regulated at the transcriptional or posttranscriptional level? For some reason, the experiments required to answer this question, such as nuclear run-on assays and sensitive RT-PCR using primers that can amplify a broad range of VSP transcripts on clones expressing a single VSP, were not reported. Moreover, for several years it was not clear whether only one VSP is transcribed from the nuclei of the parasite or whether many *vsp* genes are transcribed simultaneously and then all but one are silenced before translation, as suggested to occur for *var* genes in *Plasmodium* (18).

Several gene-silencing phenomena, including transgene silencing and co-suppression in plants, quelling in fungi, and RNA interference (RNAi) in metazoans, have many common features. Messenger RNAs (mRNAs) from the target gene are degraded into small interfering RNAs (siRNAs) by double-stranded RNA (dsRNA)-specific ribonuclease Dicer (Dcr) in a sequence-specific manner (15, 110, 150). These forms of posttranscriptional gene silencing

(PTGS) were initially described as host defense responses, in which siRNAs work as mediators of a surveillance mechanism in charge of destroying mRNAs of duplicated or aberrant genetic elements (112). On the other hand, a different class of small RNAs, called microRNAs (miRNAs), encoded within the genome of certain organisms as short hairpin structures and active during normal development, regulate expression of developmental genes by repressing the synthesis of target proteins (13, 52). Later, a variety of small RNAs were described and linked to different aspects of gene expression (27, 33, 44, 60, 66, 69). The artificial introduction of those small RNA molecules into cells that possess an active RNAi machinery specifically suppresses the expression of their homologous endogenous genes (43, 53–55, 104). In all these reports, the presence of dsRNAs is an essential aspect of the PTGS mechanism.

In 2008, it was postulated that a PTGS mechanism similar to RNAi regulates VSP expression in *Giardia* trophozoites (114). This PTGS mechanism targets the expression of a family of endogenous genes during the entire life cycle of this microorganism. Although the *Giardia* PTGS mechanism resembles gene silencing phenomena that control the expression of foreign genes, the *Giardia* PTGS mechanism targets endogenous genes.

In that study, the expression of a large number of *vsp* genes was evaluated by using suitable sets of primers that simultaneously amplify most of the *vsp* gene repertoire. These primers allowed the identification of novel *vsp* genes and were useful to determine the presence of sense and antisense VSP transcripts in different *Giardia* clones by RT-PCR. These results indicate that several sense *vsp* mRNAs, including the one that is translated, were amplified when the reverse primers were used in the RT reaction. Subsequent nuclear run-on experiments supported the simultaneous transcription of many *vsp* genes and the possibility of amplifying antisense *vsp* RNAs, excluding the one that is found by Northern blot and expressed in the trophozoite surface, when the sense primers

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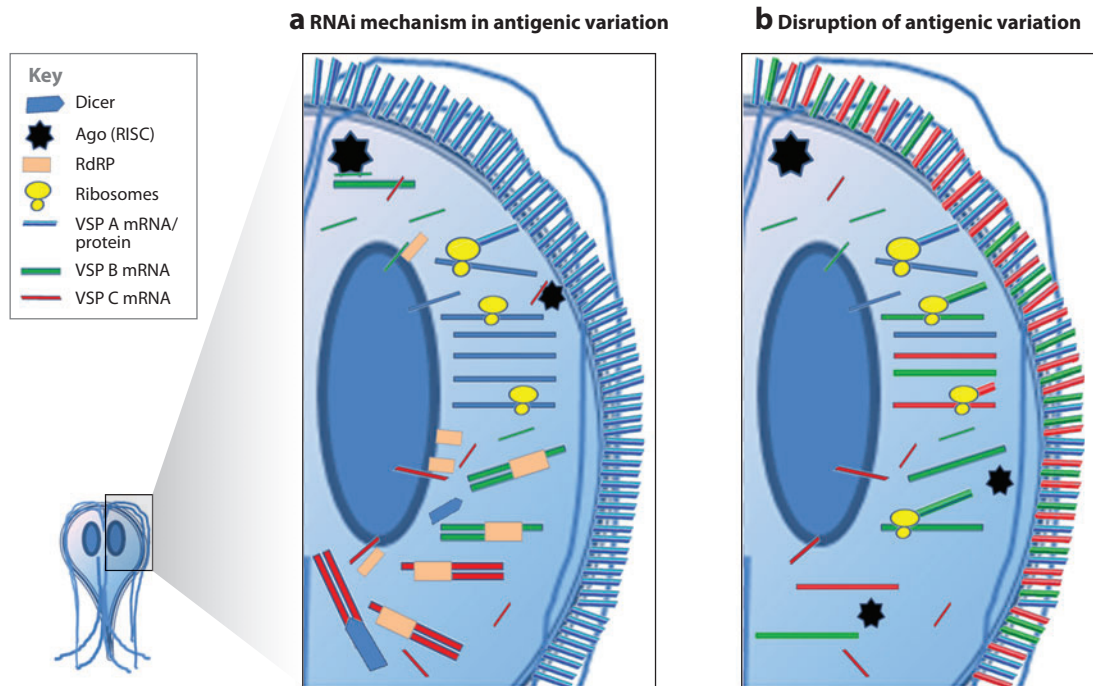
**Transcription:** the process of generating messenger RNAs from a DNA template

**dsRNA:** double-stranded RNA

**Dicer (Dcr):** an endoribonuclease RNaseIII family enzyme specific for processing dsRNA into 23- to 25-nt-long small RNAs

**PTGS:** posttranscriptional gene silencing

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**Figure 2**

Schematic of the mechanism controlling antigenic variation in *Giardia*. (a) In wild-type cells, many VSP mRNAs are generated in the nuclei of the parasite and transported to the cytoplasm. There, RdRP generates antisense VSP mRNAs, producing VSP dsRNA complexes. *Giardia* Dicer (and possible Ago) detects these dsRNAs, cleaving them into 25- to 27-nt-long small RNAs. Only one VSP transcript bypasses the silencing process by a still unknown mechanism, accumulates in the cytoplasm, and is then translated into the VSP that will be expressed on the trophozoite surface. (b) In cells in which the RNAi pathway has been disrupted (for example, Dicer's expression has been knocked down), many VSP transcripts achieve a steady-state level that allows the simultaneous expression of many VSPs on the surface of individual trophozoites. Abbreviations: Ago, Argonaute protein; dsRNA, double-stranded RNA; mRNA, messenger RNA; RdRP, RNA-dependent RNA polymerase; RNAi, RNA interference; RISC, RNA-induced silencing complex; VSP, variant-specific surface protein.

are used in the RT reaction. Nuclear run-on assays showed that many, if not all, *vsp* genes are transcribed under conditions in which only one *vsp* transcript accumulates in the cytoplasm of the parasite and is able to be translated into the protein that is then expressed on the trophozoite surface (**Figure 2a**).

A consideration about gene transcription in *Giardia* is necessary at this point. The finding of antisense *vsp* RNAs agrees with a previous report documenting the presence of long, sterile antisense transcripts in *Giardia*, which were suggested to result from a loose mechanism controlling transcription (40). Nevertheless, antisense RNAs, including *vsp* genes, were not detected in nuclear run-on assays,

suggesting that those antisense RNAs could be generated posttranscriptionally. But what is the transcriptional machinery of *G. lamblia*? *Giardia* genomes show the presence of several, but not all, molecular components involved in the synthesis and regulation of this fundamental biological process (82, 124). Are all genes constitutively transcribed in *Giardia*? No—particularly those genes that are upregulated during trophozoite differentiation into cysts (70). Although several reports indicate the activity of encystation-specific transcription factors, no definitive conclusions have been drawn (29). Is the sole presence of AT-rich regions located upstream of the initiation codon of all *Giardia* genes necessary and sufficient to



drive transcription of those genes in light of the reduced transcriptional regulatory machinery of the parasite (82)? Nevertheless, exquisite metabolic control must exist given the expensive energetic cost of synthesizing many transcripts that will be silenced, at least for VSPs.

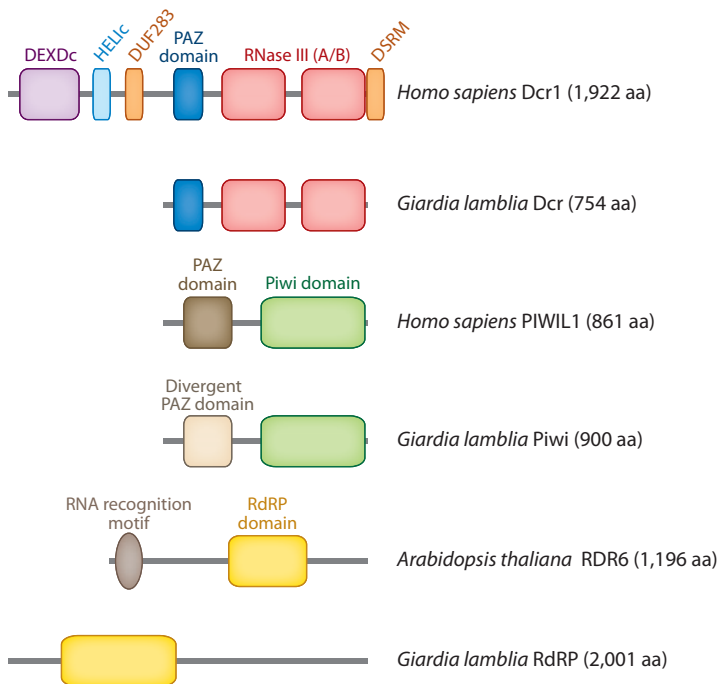
### GIARDIA POSTTRANSCRIPTIONAL GENE SILENCING MACHINERY

An RNA-dependent RNA polymerase (RdRP) homolog that localizes to the rough endoplasmic reticulum of trophozoites has been described (113, 114). RdRPs are expected to have a cytoplasmic or nuclear localization (9); however, immunolocalization results show that gRdRP is likely associated with ribosomes on the cytoplasmic side of the rough endoplasmic reticulum. These results suggest a participation of RdRP in cytoplasmic events preceding translation at polyribosomes, where a screening process detects homologous *vsp* mRNAs and transforms them into dsRNAs. The direct involvement of RdRP in gene silencing phenomena induced by transgenes has been shown, and dsRNAs produced by RdRP have been suggested to be necessary intermediates of the silencing process (30, 109). These results support the model predicting that the presence of multiple *vsp* transcripts in *Giardia* directs the generation of antisense RNAs by RdRP. Therefore, what are the structural characteristics of *Giardia* RdRP that make this enzyme active only in the presence of homologous VSP transcripts (114)? Are antisense transcripts for many other genes (40) also generated by this enzyme? If so, how does RdRP select the aberrant transcripts for synthesizing antisense RNAs? Or, because these antisense transcripts as described by others (40) are not evident in nuclear run-on assays (114), could they be artifacts of library construction? These are relevant questions that require further experimentation to fully understand gene regulation in *Giardia*.

After the synthesis of antisense VSP mRNA transcripts by *Giardia* RdRP, it was suggested

that the produced *vsp* dsRNAs trigger the action of an endonuclease complex that processes these molecules into short 25-nt-long dsRNAs in an ATP-dependent fashion, a feature of RNA-silencing systems (103, 138). In addition to RdRP, different enzymes involved in processing dsRNAs in other eukaryotes, including modules of Dicer (Dcr) (a bidentate RNase III and many ATP-dependent DEAD-box RNA-helicases) and Piwi/argonaute (Ago), were identified in *Giardia* and were constitutively expressed in trophozoites (114). Because *Giardia* belongs to the earliest diverging branch of the eukaryotic line of descent, the presence of separated genes, in which each gene carries out a single task instead of one gene performing all tasks (as in higher eukaryotes), is not surprising (Figure 3). However, because *Giardia* Dcr is the smallest Dcr known so far, it was used to obtain the first three-dimensional structure of

**RNA-dependent RNA polymerase (RdRP):** an enzyme that generates antisense RNA using aberrant RNAs as a template  
**Ago:** Argonaute protein



**Figure 3**

Diagram of the component of the RNAi machinery of *Giardia lamblia*. Schematic illustration of the different domains of RdRP, Dicer, and Piwi/argonaute of *G. lamblia* compared with similar molecules of higher eukaryotes. Abbreviation: RdRP, RNA-dependent RNA polymerase.

this class of enzymes and was found to be active even in the absence of the RNA helicase domain (72–74). This domain has been reported to confer specificity and to control activity of Dcr in higher organisms (72–74). Therefore, this finding must explain why *Giardia* Dcr is able to cleave any dsRNA (synthetic or endogenous) in vitro (74, 114). Experiments in vivo consisting of either the introduction of exogenous small dsRNAs into the cells or the endogenous production of small dsRNAs are expected to induce a strong RNAi response. It was recently reported, however, that the *Giardia* RNA pathway is active only against long and specific dsRNAs (117), which contradicts previous studies performed by other groups (74, 114).

The molecular components of *Giardia* silencing machinery show homology to those of higher organisms, suggesting a similar function in this primitive cell (114). Direct evidence for the participation of RdRP and Dcr in the regulation of VSP expression in *Giardia* was obtained by generating transfectant cells that constitutively express Dcr and RdRP antisense transcripts (114). Knockdowns of any of these enzymes resulted in an alteration of the VSP expression pattern such that more than one VSP was produced by individual trophozoites, demonstrating the essential role of these molecules at different steps of the silencing process (**Figure 1c** and **Figure 2b**).

## MECHANISMS OF ANTIGENIC SWITCHING

Given that this endogenous RNAi system is involved in the control of antigenic variant expression in *Giardia*, how does a single *vsp* transcript bypass this silencing process? In some organisms, siRNAs correlate with methylation of homologous DNA sequences, which in turn modulates transcription (32, 105, 109); however, DNA methylation has not been demonstrated in *G. lamblia* (2, 114). Hence, other nuclear modifications, such as variations in chromatin structure linked to the cell cycle, might produce differences in transcription

efficiency, as reported for other parasitic organisms (12, 19, 80, 106).

After transcription of the entire *vsp* repertoire, differences in the level of individual transcripts might serve as the exclusion factor that allows the *vsp* mRNA with the highest concentration to evade the action by the *Giardia* RdRP and subsequent degradation by the silencing machinery (114). Because components of the RNAi machinery, including Piwi/Ago family members, have been involved in histone modifications and transcriptional derepression in other organisms, Ago might well be required for a similar function in *Giardia*. However, silencing of Ago resulted in unviable cells, indicating that it may have an additional function in *G. lamblia* (114), i.e., controlling the integrity of the genome by blocking the activity of transposons (139). RdRP could be involved in some cellular mechanisms operating after transcription to recognize highly homologous mRNAs by scanning the entire *vsp* transcriptome before initiating translation at the ribosomes. RdRP can generate antisense RNA only when homologous transcripts are present, acting as a concentration-dependent sensor of aberrant mRNAs (150). This hypothesis was supported further by experiments indicating that affinity-purified HA-tagged *Giardia* RdRP generates high-molecular-weight RNA in vitro only when more than one *vsp* transcript is present in the mixture (114). Attempts to silence either the VSP being expressed or the overexpression of a different VSP, under the control of a strong promoter, do not influence antigenic variation, and continuous switching occurs even under these circumstances (114). Similar results were reported when a DNA fragment containing the *vspH7* gene tagged with an HA epitope was integrated into the GS genome (65). Trophozoites could express only the native *vspH7* gene and not the HA-tagged *vspH7*, even when the upstream and downstream regions as well as the coding regions are virtually identical. These results indicate that the control of VSP expression is likely independent of the promoter that drives its expression, and that another

mechanism(s), either postnuclear or epigenetic, must be regulating antigenic variation in *Giardia*.

These results are consistent with a major role for the cellular PTGS machinery in the selection of the *vsp* transcript destined to be expressed in any single trophozoite (**Figure 2a**). Nevertheless, how *Giardia* changes VSPs on their plasma membrane is still an unresolved question. Because VSP switching occurs randomly, we favor the hypothesis that variations in either the general or local concentrations of different VSP transcripts may determine which transcript will circumvent the silencing system, as was suggested to occur in higher eukaryotes (45, 61, 62). It is then possible that during cell division each daughter cell receives varying amounts of different *vsp* transcripts (or *vsp* siRNAs), permitting a switch to the expression of multiple VSPs (114). Alternatively, there may be differences in the level of transcription for each *vsp*. Consistently with this hypothesis we have detected variations on individual *vsp* expression levels in nuclear run-on experiments due to differences in the level of histone modifications. This sort of variation seems to depend on the chromatin state in different areas of the genome or on the position of each gene within the genome.

In another report, a posttranscriptional mechanism involving miRNAs was associated with the control of the expression of 22 genes from the VSP repertoire of isolate WB (121). In a previous work, small nucleolar RNAs (snoRNAs) were identified, analyzed, and used to search for possible targets in the *Giardia* genome on the basis of previously identified snoRNAs from other organisms (143). In addition, these authors identified putative miRNA recognition sequences and evaluated the involvement of two key enzymes, Dcr and Ago, in the RNA-mediated silencing mechanism. The results presented by these authors indicated that the original miRNAs are involved in transcription repression of several genes, including only 22 *vsp* genes (121). Despite the important discovery that miRNAs can be generated from

snoRNAs (63), no direct evidence of the involvement of miRNAs produced in gene regulation was provided. Nevertheless, modifications in the concentration of the VSP mRNA level, in transcription repression (transcriptional gene silencing, or TGS), in transcript degradation, or in VSP mRNA translation may be playing a role in the fine control of the regulation of VSP expression.

From the current knowledge on most mechanisms that control gene expression, it is evident that no single biochemical pathway can act on its own. A cross-talk between different machineries may define the fate of an expression event in eukaryotic cells. Although there have been important advances in *Giardia*, there is still a long way to go before we elucidate the process that drives the expression of a unique VSP on the surface of the trophozoites and, yet more relevant, how a VSP is replaced by another VSP. Is this switching occurring randomly or is it linked to the general metabolism of the cell? On the basis of results from experiments in which mAbs were added to the parasite culture, it seems that those antibodies are involved in triggering antigenic switching (134, 135), but this event takes place even in the absence of immunologic pressure (92). Is this a genetically codified process or is a stimulus necessary to initiate the exchange of surface antigens?

## ANTIGENIC VARIATION IN VIVO AND HOST IMMUNE RESPONSES

Antigenic variation is thought to be used by *Giardia* to (a) survive the hostile environment of the upper small intestine (100), (b) diversify to infect a wide range of hosts (92), and (c) evade the host immune system (91). *Giardia* infections are characterized by a variety of symptoms and clinical courses. Chronic or recurrent infections occur and antigenic variation has been suggested as responsible for prolonged infections (91, 92). In general, the production of immunologically distinct variants affects the dynamics of infections within their host, enhancing

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**TGS:** transcriptional gene silencing

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parasite persistence in a hostile immune response, prolonging the infection, and increasing the transmission of the disease among susceptible hosts (35).

Earlier works were designed to determine if two well-characterized *Giardia* isolates cause infection and disease in humans. Nash and coworkers (96, 97) infected humans with the GS/M and Isr (similar to WB) isolates. In these important studies, the fulfillment of Koch's postulates and *Giardia* pathogenicity were demonstrated in humans for the first time. The results demonstrated that some individuals may be chronically infected, others may be re-infected with or without symptoms, and others do not develop a protective immune response during the initial infection, although serum and intestinal anti-*Giardia* antibodies (particularly IgA) were detected (96, 97). Therefore, the characteristics of particular infections in humans seem to be dependent on a variety of factors (20, 21, 125).

For this reason, the use of laboratory animals is required to overcome the difficulties observed in human patients (8, 11, 25, 50, 127). Most studies on giardiasis have focused on murine models of infection to analyze the immune responses during the course of the infection. Normal adult mice can be easily infected with *G. muris*. *G. muris* infections in adult, immunocompetent mice are self-limiting, as are most human infections with *G. lamblia* (115, 119), making this model a valuable tool to define immune defenses against *Giardia* (41). However, some individuals (142) and immunodeficient mice (118, 119) have prolonged infections, presumably because of antigenic variation (91). Although many studies were performed with the murine parasite, there are several disadvantages in using *G. muris*: It cannot be grown in vitro, it does not infect humans, and, until recently, it was unknown if it also undergoes antigenic variation (120). For these reasons, although the immune response could be easily determined, the influence of antigenic variation during the course of the infection was not considered (41). Nevertheless, the studies in mice provided useful

information regarding the immunology of giardiasis.

An important animal model for giardiasis is the Mongolian gerbil (*Meriones unguiculatus*). Adult gerbils can be infected with different strains of *G. lamblia* (7, 140), and parasites undergo antigenic variation in this host (84). In this model, infections are associated with disease symptoms, including diarrhea and weight loss (22, 36). Unfortunately, although the gerbil is a valuable model to study the mechanisms of *Giardia*-induced intestinal disease, the genetic information on the immune system of gerbils is limited owing to the lack of immunologic reagents. Nonetheless, this model has been used successfully to verify antigenic variation during the course of infection (91).

All these studies in animal models showed that immune responses to *Giardia* are characterized by a strong response to VSPs (91). Given that parasite protection against specific immune responses depends on switching the expression among immunologically distinct VSPs, hosts might prevent infections with specific immunological responses directed to all surface antigens.

## DISRUPTION OF ANTIGENIC VARIATION IN AN ANIMAL MODEL OF INFECTION

To analyze the hypothesis that trophozoites expressing the entire repertoire of VSPs on their surface are able to confer protection from subsequent infections (**Figure 1c** and **Figure 2b**), experiments using the *Giardia* WB isolate in the gerbil model of giardiasis were performed in a series of well-controlled tests that overcame the deficiencies of previous models (e.g., animals free from previous *Giardia* or related parasite infections, identical flora in all animals, and animals that reproduce the symptoms observed in human infections) (116). This was the first time that organisms in which the mechanism of antigenic variation had been disrupted were used to determine not only the course of infection but also the host response to the parasite. The results showed that initial infection with cells

expressing all VSPs encoded in their genome highly protected the animals from subsequent infection by *Giardia* clones expressing a unique VSP on their surface or by cysts. In addition, immunization of gerbils with VSPs purified from these cells using a mAb targeting the 5-amino-acid conserved cytoplasmic tail also conferred protection against secondary infections in this experimental model. An inflammation of the gerbil upper small intestine with a significant increase in the size of Peyer's patches and mononuclear infiltration of the mucosa was noticed during *Giardia* infections with wild-type and transgenic trophozoites (116). Notably, none of these histological changes was observed when the gerbils were vaccinated orally with the entire repertoire of purified VSPs, indicating that VSPs are not toxic to the animals, which, unlike infected animals, did not manifest any signs of the disease (116). Because previous reports suggested that VSPs share characteristics with toxin homologs (31) and because the results show that VSPs cause neither inflammation nor pathological signs in gerbils, it is clear that the disease is caused by the whole trophozoite and not by its surface coat (116).

Early studies reported that axenically cultured trophozoites of *G. lamblia* were killed in vitro by human sera containing antiparasitic antibodies. In these studies (56), the mortality of trophozoites induced by human serum and complement varied from 10% to 98%. However, it was not clear from the experiments whether this variability was due to parasite diversity or to different anti-VSP antibodies present in the human sera. In addition, studies performed with *G. muris* trophozoites demonstrated that sera from both resistant and susceptible mice were able to lyse or immobilize trophozoites in vitro (16). In many studies, immobilization of trophozoites was reported to be an important part of the host's defense system against *Giardia*. It is known that continuous flagellar movement is required for successful attachment of trophozoites to substrates (42). Immobilization of trophozoites in the lumen of the small intestine would result in the inability of the organism to attach to the epithelium and

eventual elimination of trophozoites from the gut. For these reasons, VSPs are the principal target for the generation of protection against *G. lamblia*.

Additional results by Rivero et al. (116) showed that sera and intestinal content obtained from animals infected with trophozoites expressing the full repertoire of VSPs were able to agglutinate the trophozoites, indicating the presence of antibodies against VSPs in those fluids. These results showed, for the first time in any parasite, direct experimental evidence that (a) antigenic surface variation is an essential mechanism responsible for evading host immune responses, (b) variable surface antigens are crucial for the establishment of the infection in a host, and (c) immunization with the whole repertoire of variable surface antigens protects the host against secondary infections.

Overall, these results indicate that either an original infection or primary immunizations with VSP preparations are able to confer the animals with an immune response capable of preventing subsequent infection with *Giardia* clones or cysts obtained from the stool samples of infected individuals, showing the important role of VSPs in the establishment of the infection. Vaccination with the whole repertoire of VSPs demonstrates the role of antigenic variants in evading the host immune system and confirms the importance of antigenic variation as an adaptive mechanism developed by parasites to cause chronic and recurrent infections. These results pave the way for generating a vaccine not only against *Giardia* but also against other parasites that undergo antigenic variation (116).

In summary, it was demonstrated that disruption of antigenic variation in *G. lamblia* can generate viable parasites that complete their life cycle in vivo and in vitro and that are capable of infecting animals and conferring protection against subsequent infections (116). Similarly, oral immunization with the entire repertoire of variable surface antigens prevented the establishment of infection by trophozoites or cysts (116). Because many pathogenic microorganisms also use

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**Vaccine:** an antigenic composition that once administered to an individual protects it from infections

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antigenic variation to evade host immune responses, avirulent strains or parasites in which the molecular mechanism of antigenic variation could be deregulated to allow the expression of multiple variants might be a helpful tool for the development of vaccine

formulations against important human pathogens. The demonstration of this proof of principle in *Giardia* allows a redefinition of the current strategies of immunoprophylaxis against other parasites that present antigenic variation.

### SUMMARY POINTS

1. *G. lamblia* is a protozoan parasite that colonizes the upper small intestine of humans and many vertebrate hosts.
2. *Giardia* parasites undergo antigenic variation by the continuous switching of VSPs.
3. VSPs are type 1a integral membrane proteins that cover the entire surface of the parasite. VSPs are composed of a variable, cysteine-rich N-terminal extracellular region and a highly conserved C-terminal region, including a unique transmembrane domain and a short, 5-amino-acid-long cytoplasmic tail.
4. The *Giardia* genome contains a repertoire of approximately 200 VSP-coding genes, but only 1 VSP is expressed at any given time on the surface of the parasite.
5. Switching of one VSP to another VSP occurs once every 6 to 16 generations, even in the absence of immunological pressure.
6. The mechanism that controls antigenic variation in *Giardia* resembles the RNAi pathway; it involves RdRP, Dcr, and Piwi/Ago enzymes.
7. Disruption of antigenic variation in *Giardia* by silencing the expression of components of the RNAi machinery leads to the expression of multiple VSPs in individual cells.
8. Infection with cells in which antigenic variation has been disrupted or oral immunization with VSPs purified from these transgenic cells fully protects animals from subsequent *Giardia* infections.

### DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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**71. Describes the biochemical characteristic of the first VSP purified from trophozoites, including its metal binding capabilities.**

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**74. Shows, for the first time, the structure of a Dicer enzyme using *Giardia* Dicer as a model to determine the basis for dsRNA cleavage.**

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**82. Describes the results of the completion of the first *Giardia* genome ever sequenced and shows the minimalistic genomic organization of this parasite.**

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114. Shows the first demonstration of regulation of a family of homologous genes by RNAi.

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116. Shows the first experimental evidence that antigenic variation is crucial for evading host immune responses.

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